
ORIGINAL ARTICLE

Impact of Aging

*Sporadic, and Genetic Risk Factors
on Vulnerability to Apoptosis in Alzheimer's Disease*

**Katharina Schindowski,¹ Tilmann Kratzsch,² Jürgen Peters,²
Barbara Steiner,¹ Silke Leutner,¹ Natalie Touchet,³
Konrad Maurer,² Christian Czech,^{3,4} Laurent Pradier,³
Lutz Frölich,² Walter E. Müller,¹ and Anne Eckert^{*,1}**

¹Department of Pharmacology, Biocenter, J.W. Goethe University of Frankfurt, Marie-Curie-Str. 9, D-60439 Frankfurt am Main, Germany; ²Department of Psychiatry and Psychotherapy I, J.W. Goethe University of Frankfurt, Heinrich-Hoffmann-Str. 10, D-60528 Frankfurt am Main, Germany; ³Aventis Pharma, Research and Development, 13 Quai Jules Guesde, F-94403 Vitry-sur-Seine, France; ⁴Present address: F. Hoffmann-La Roche AG, CNS Research, CH-4070 Basel, Switzerland

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Abstract

The identification of specific genetic (presenilin-1 [PS1] and amyloid precursor protein [APP] mutations) and environmental factors responsible for Alzheimer's disease (AD) has revealed evidence for a shared pathway of neuronal death. Moreover, AD-specific cell defects may be observed in many other nonneuronal cells (e.g., lymphocytes). Thus, lymphocytes may serve as a cellular system in which to study risk factors of sporadic, as well as genetic AD in vivo. The aim of our present study was to clarify whether lymphocytes bearing genetic or sporadic risk factors of AD share an increased susceptibility to cell death. Additionally we examined whether a cell type-specific vulnerability pattern was present and how normal aging, the main risk factor of sporadic AD, contributes to changes in susceptibility to cell death. Here, we report that lymphocytes affected by sporadic or genetic APP and PS1 AD risk factors share an increased vulnerability to cell death and exhibit a similar cell type-specific pattern, given that enhanced vulnerability was most strongly developed in the CD4⁺ T-cell subtype. In this paradigm, sporadic risk factors revealed the highest impact on cell type-specific sensitivity of CD4⁺ T cells to apoptosis. In contrast, normal aging results in an increased susceptibility to apoptosis of both, CD4⁺ and CD8⁺ T cells.

Index Entries: Transgenic mice; presenilin-1; amyloid precursor protein (APP); lymphocytes; AD patients; apoptosis.

*Author to whom all correspondence and reprint requests should be addressed. E-mail: A.Eckert@em.uni-frankfurt.de

Introduction

Apoptosis is of particular interest in aging, as it is thought to play an important role in various age-related neurodegenerative diseases. Alzheimer's disease (AD) is the leading cause of age-related dementia, characterized by deposition of β -amyloid ($A\beta$) plaques, accumulation of intracellular neurofibrillary tangles, and pronounced neuronal cell loss. Importantly, aging represents the main risk factor of sporadic AD. About 10% of AD patients suffer from an autosomal dominant inheritable variant of AD. Three genes are causatively linked to the pathogenesis of early-onset familial AD (FAD) forms. In addition to the genes encoding for PS1 on chromosome 14 and presenilin-2 (PS2) on chromosome 1, mutations in the APP gene on chromosome 21 account for FAD cases. Despite the fact that neuronal death is a central lesion in AD, the mechanism of how FAD-linked mutants cause neuronal cell death is still not clearly understood. One potential mechanism underlying the neurotoxicity of these FAD mutants is the generation of amyloid- β ($A\beta$), a 39- to 43-amino acid long peptide representing the major constituent of senile plaques in the brains of AD patients, owing to an altered proteolytic processing of APP. Although significant progress has been made in our understanding of the proteolytic processing of APP and the secretion of $A\beta$ peptide, the mechanisms for the accumulation of $A\beta$ deposits and their role in AD pathogenesis remains a matter of speculation (Glabe, 2000; Sambamurti et al., 2002).

Furthermore, there is growing evidence that disturbances in the tight regulation of apoptosis may contribute to the progressive neuronal loss in AD brain (Anderson et al., 1996; Cotman, 1998; Stadelmann et al., 1999). Several recent findings have linked $A\beta$ toxicity (Loo et al., 1993) and increased $A\beta$ production resulting from FAD-related APP or PS1 mutations to cell death (Czech et al., 2000; Mattson et al., 2001; Selkoe, 2001). Recent findings indicate that the expression of mutant PS1 or mutant APP in PC12 cells sensitizes cells to apoptosis (Guo et al., 1997; Eckert et al., 2001c; Marques et al., 2003). Furthermore, cultures of primary neurons from PS1 mutant knock-in mice (PS1 M146KI) and from transgenic mice bearing human mutant PS1 M146L show increased vulnerability to cell death (Guo et al., 1999a; Guo et al., 1999b). In addition, lymphocyte cell lines (e.g., the human Jurkat T-cell line), trans-

fectured with mutant PS1 (Wolozin et al., 1998), and lymphocytes from FAD patients bearing PS1 mutations (Parshad et al., 1996) show similar results in regard to increased vulnerability to DNA damage leading to accelerated cell death. Moreover, enhanced vulnerability to cell death has also been described in peripheral lymphocytes from AD patients (Hartwig, 1995; Parshad et al., 1996; Eckert et al., 1998; Eckert et al., 2001a) but not in patients with vascular dementia (Hartwig, 1995; Eckert et al., 2001a). Oxidative stress mechanisms seem to influence this process (Gibson et al., 2000; Eckert et al., 2001a; Mecocci et al., 2002). In order to close the gap between studies using neuronal cell lines transfected with PS1 mutations, neurons from mutant PS1 transgenic animals, and peripheral cells of AD patients, we recently investigated lymphocytes from mice transgenic for FAD specific PS1 mutations. Importantly, lymphocytes from mice bearing mutant PS1 exhibited a similar hypersensitivity to apoptosis as do lymphocytes from AD patients (Eckert et al., 2001b). Thus, this animal model of AD successfully mimics common alterations in sensitivity to cell death as seen in neurons and peripheral cells from AD patients.

Analyses of the distribution of pathological changes in AD suggest that this disease affects selected populations of neurons within the cerebral cortex, whereas other neuronal subgroups are remarkably resistant to the degenerative process. Currently, no clear evidences for a predominant expression of APP or PS1 in the affected neurons, which might contribute to the cell type-specific vulnerability, have been provided. Several brain regions (e.g., hippocampus and cerebellum) express high levels of APP or PS1 within their neurons (Arai et al., 1991; Page et al., 1996; Mathews et al., 2000) but are affected differently by neurodegeneration. Based on these findings, we investigated the vulnerability of specific subgroups of peripheral Alzheimer's cells to cell death and the susceptibility pattern in sporadic and familial forms of the disease. It would be helpful to have a peripheral model (e.g., similar types of cells originating from the same individual with either sensitivity or resistance to apoptotic cell death). For this reason, we included in our study—in addition to sporadic AD patients—the use of young transgenic mice (3 mo old) bearing Alzheimer-specific mutations (shown to be suitable for this experimental attempt) (Eckert et al.,

2001b) and investigated the sensitivity threshold for the induction of apoptosis in T-cell subpopulations. In addition, we included transgenic mice bearing either mutant APP or mutant A β PH, and mutant PS1. Moreover, age-related elevations of apoptotic cell death were detected in human and murine lymphocytes (Schindowski et al., 2000). Studying lymphocytes from young mice bearing FAD mutations has the advantage to investigate solely the impact of genetic AD factors on cell death vulnerability by excluding potential effects raised by aging. Our attempt to search for cell type-specific alterations in the cell death sensitivity of lymphocytes affected by aging, sporadic, or genetic risk factors may help to differentiate common and divergent mechanisms of changes in the susceptibility to apoptosis in aging and sporadic AD, and in sporadic and familial AD.

Methods

Human Subjects

Twenty-six patients (9 men and 17 women) with AD were studied. Dementia was diagnosed according to ICD-10. Diagnosis of Alzheimer's disease was achieved following the guidelines of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders (NINCDS-ADRDA criteria) task force. Furthermore, a MRI scan for structural imaging and HMPAO-SPECT or FDG-PET for functional neuroimaging were included. The mean age was 70.9 ± 9.1 yr (range 52 to 89 yr). The majority of cases exhibited mild to moderate dementia. The mean Mini Mental State Examination (MMSE) score was 18.6 ± 5.6 and the mean Global Deterioration Score (GDS) was 4.1 ± 1.0 . FAD patients could be excluded by patient history. Blood cells from 21 nondemented individuals of similar age (mean age 73.2 ± 7.5 yr, range 60 to 85 yr, 8 men and 13 women) were used as controls (average MMSE 29 ± 0.5). As a result of methodological reasons, only a subgroup of patients and sex- and age-matched nondemented controls ($n = 12$, 4 men and 8 women; mean age 69.8 ± 6.6 yr) was available for stimulation experiments with lymphocytes. In addition, young subjects (4 men and 7 women; age under 35 yr, mean age 25.4 ± 5.1 yr) were included within the study. Majority of AD patients and aged controls were treated with drugs,

primarily for cardiovascular disease, but did not receive medications known to interact with lymphocyte functions, or psychotropic medication. Subjects with acute infections or immunological alterations according to the SENIEUR protocol (Ligthart et al., 1984) were not included in the study. The study was approved by the responsible Ethical Committee and written informed consent from all subjects was received, where appropriate, from their caregivers.

Generation and Characterization of Transgenic Mice

Generation and expression analysis of transgenic mice was described in detail previously (Leutner et al., 2000; Wirths et al., 2001). In brief, the coding regions of human wild-type PS1 (PS1 wt) or PS1 M146L were introduced into a genomic construct with the promotor of the murine 3-hydroxy-3-methyl-glutaryl CoA reductase gene (HMG-CR) that shows a strong and ubiquitous expression pattern in peripheral tissues and in the brain (Leutner et al., 2000). The generation of PDGF β -APP 695 SDL mice has been described in detail previously (Wirths et al., 2001). Three different FAD mutations (SDL: Swedish mutation KM670/671NL, Dutch mutation E693Q, London mutation V717I) have been introduced in the human APP695 sequence. APP expression was under the control of PDGF β promotor, which induces clear expression of human APP in lymphocytes from APP695SDL mice. Additionally, double transgenic mice were generated by crossing PS1 M146L with APP695SDL mice. Transgenic ($n = 6$ for all groups) and nontransgenic age and sex-matched control animals ($n = 6$) were employed at an age of 3 mo. Animals were handled according to French and German guidelines for animal care.

Isolation of Lymphocytes

Blood from patients and control subjects was collected between 8:00 and 10:00 AM to avoid influence by circadian rhythm. Peripheral blood lymphocytes were separated from heparinized blood by centrifugation on Ficoll-Hypaque density gradient for 400g for 40 min as described previously (Schindowski et al., 2000).

Because large quantities of T cells have to be obtained from each animal for our experimental

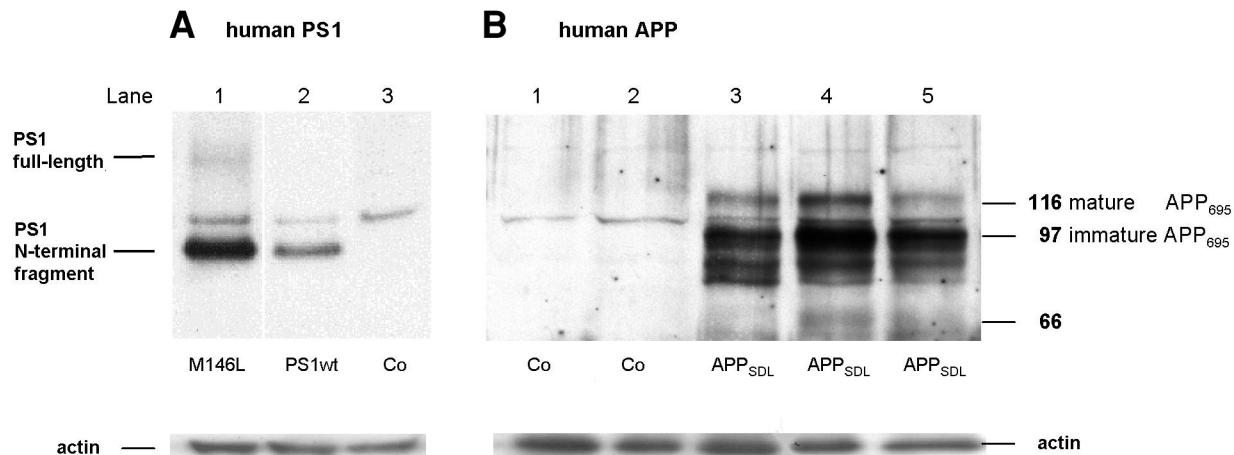


Fig. 1. Expression of human APP and human PS1 in transgenic mice. **(A)** Western blot analysis of PS1 expression (50 μ g protein per lane). Expression of human PS1 is not observed in T lymphocytes from nontransgenic control mice (*lane 3*). The amino-terminal fragment of PS1 is detectable in all groups of transgenic mice (*lane 1* = PS1 M146L; *lane 2* = PS1wt) whereas full-length PS1 is only slightly visible in mice expressing mutant PS1 (*lane 1*). Representative Western blot analysis of lymphocytes from individual mice is shown. Immunoblotting with an anti-actin antibody (*lower panel*) confirmed equal protein loading. **(B)** Intracellular APP from cell lysates of T lymphocytes. Equal amounts of protein (50 μ g per lane) were loaded on a 8% SDS-polyacrylamide gel. Multiple bands reflect different splice isoforms and different posttranslational modifications of APP (indicated on the right). T lymphocytes from transgenic mice bearing mutant APP695SDL (*lanes 3–5*) show clear expression of human APP in contrast to nontransgenic control mice (*lanes 1 and 2*). As a control, the blot was probed with an actin antibody (*lower panel*).

design, lymphocytes were isolated from the spleen instead of circulating blood. In a previous study, we demonstrated that murine lymphocytes from blood and spleen behave similar in respect to cell death vulnerability (Eckert et al., 2001b). Spleen cell suspensions were prepared by mechanical dissociation of individual murine spleens. Red blood cells were lysed in hypotonic buffer as described previously (Eckert et al., 2001b).

Expression of Human PS1 and Human APP

For Western blotting, protein extracts from T lymphocytes isolated from the spleens of transgenic (PS1wt, PS1 M146L, APP695SDL) or nontransgenic littermate control mice were prepared. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), PS1 proteins were identified by immunoblotting using the human-specific antibody MAB1563 (Chemicon) as described previously (Leutner et al., 2000) (Fig. 1A). For human APP, the lysate was separated on an 8% SDS-polyacrylamide gel.

The gel was blotted into nitrocellulose membrane and human APP was detected with polyclonal anti-serum 5313 recognizing only human APP (Fig. 1B).

Induction of Apoptosis

Cells were investigated for apoptosis directly after isolation (termed basal in vitro apoptosis). Cells were plated in 48-well plates at a density of 5×10^5 cells/mL and were incubated in the presence or absence of a cell death-inducing agent (2-deoxy-D-ribose, d-Rib, Sigma, 10 mmol/L) for 24 h.

Antibodies and Dyes for Flow Cytometry

The mouse monoclonal antibodies specific for human antigens used in this study included: anti-CD3 (clone SK7), anti-CD19 (clone 4G7), anti-CD4 (clone SK3), anti-CD8 (clone SK1) and isotype control IgG₁ (clone X40). Monoclonal antibodies specific for murine T cell subsets: anti-mouse CD3 (145-2C11), anti-mouse CD4 (L3T4)(RM4-5), anti-mouse CD8 (Ly-2) (53-6.7). All antibodies were obtained from

Becton Dickinson/Pharmingen, Heidelberg, Germany. 7-Aminoactinomycin D (7-AAD) was purchased from Molecular Probes (Leiden, The Netherlands).

Staining of Whole Blood for the Detection of Subset Distribution

Whole blood was stained with the appropriate antibodies following the manufacturer's manual. Samples were analyzed using Simulset software (BD). Forward (FSC) and side (SSC) angle scattered light-gating excluded debris, granulocytes, and monocytes. For each sample, 10,000 events were acquired.

Quantification of Total Lymphocyte Number in Whole Blood Using TrueCount™

Absolute number of lymphocytes were determined with TrueCount beads (BD: Lot 60158, counts 46900) and CD3-PerCP, CD8-PE, and CD4-FITC according to the manufacturer's manual using TrueCount Absolute Count Tubes (Becton Dickinson, Germany). Absolute cell number was calculated using the following equation: no. of events in cell population region (CD3, CD4 or CD8)/no. of events in bead region \times 46900 beads/test volume (50 μ L) = cells/ μ L

Detection of Apoptosis in Lymphocyte Subsets by Flow Cytometry

The actinomycin D analog, 7-AAD, is able to bind to GC regions of the DNA. During the cell death process, the plasma membrane becomes permeable to dye. In addition to readily quantifying apoptosis, 7-AAD staining offers the advantage of discriminating between early and late apoptotic cells. Staining with 7-AAD can be combined with other staining procedures to study apoptosis in a multiparametric approach at a single cell level (Lecoeur et al., 2002). Lymphocytes were triple stained with FITC-conjugated anti-CD4 (FL-1 channel), PE-conjugated anti-CD8 antibody (FL-2 channel), and the nuclear dye 7-AAD. 1 μ L of each antibody and 7-AAD (6 μ mol/mL) (Schmid et al., 1994) were added to the 48-well plates for 20 min at room temperature. Cells were harvested, washed twice with PBS, resuspended in CellWASH™ (Becton Dickinson, Germany), and kept in the dark before flow

cytometry analysis. Debris and monocytes were excluded by Forward (FSC) and Side (SSC) angle scattered light gating. A total of 10,000 individual nuclei per measurement was analyzed in each experiment providing valid quantitative data. For determination of apoptotic cells, two-parameter dot plots (FITC, PE) showing surface antigens staining (CD4-FITC and CD8-PE) were then created and each subset was marked with a distinct color. Gated cells were displayed in a new dot plot showing FSC plotted against 7-AAD (FL-3 channel). Living, early, and late apoptotic cells were defined according to the level of 7-AAD staining (labeled "live," "apoptotic" and "dead" within dot plots) (Lecoeur et al., 2002).

Statistics

Data are given as mean \pm SEM. For statistical comparison, Student's *t*-test, one-way ANOVA followed by Tukey's post hoc test or two-way ANOVA were utilized. *p* Values less than 0.05 were considered to be statistically significant.

Results

Expression of Mutant Alzheimer's Genes in Lymphocytes From Transgenic Mice

The transgenic mice used in the present study have been previously characterized for expression of the human transgene in their brains (Leutner et al., 2000; Wirths et al., 2001). To study effects of genetic FAD risk factors on cell death vulnerability in lymphocytes, these cells have to meet the basic requirements of expression of mutant APP and/or PS1 that appear to be ubiquitously expressed throughout the human body. Transgene expression of human PS1 is under control of a modified HMG-CoA reductase promoter. This housekeeping-type promoter shows a strong and ubiquitous expression pattern with high expression in neurons (Leutner et al., 2000). In order to assess human PS1 expression, we performed Western blot analysis of cell lysates from T lymphocytes of transgenic mice and nontransgenic littermate controls. The Western blot was probed with a human-specific monoclonal antibody (MAB1563, Chemicon) directed against the amino-terminal part of PS1 for the detection of full-length PS1 and N-terminal fragments. This antibody is specific for human PS1, because no signal

of the endogenous PS1 is detectable in the lysate of the nontransgenic mice (Fig. 1A, lane 3). Transgenic mice bearing either human PS1 wt or mutant PS1 M146L showed clear expression of the transgene in their T lymphocytes (Fig. 1A, lanes 1 and 2). In accordance with our previous findings in the brains of PS1 transgenic mice (Leutner et al., 2000), expression of human PS1 was higher in PS1 M146L mice compared to PS1 wt mice and small amount of full-length PS1 was detectable in PS1 M146L mice (Fig. 1A, lane 1). Given that the comparison of two different mutant PS1 transgenic lines with either low or high expression of human PS1 to the wt PS1 line showed that mutations in PS1, and not simple overexpression of the transgene, caused alterations in the vulnerability to cell death (Eckert et al., 2001b), PS1 expression level may not be responsible for the cellular changes described in the following section.

Transgene expression of human APP is under control of the PDGF promoter (Wirths et al., 2001). For detection of human APP, the human-specific rabbit antiserum 5313 was used (Eckert et al., 2001c). As expected, in mice bearing the human APP transgene, Western blotting revealed a clear expression of the transgene in lymphocytes from transgenic mice (Fig. 1B, lanes 3–5), whereas the antiserum did not recognize any APP species in the nontransgenic littermate controls (Fig. 1B, lanes 1 and 2).

Thus, we could demonstrate a clear expression of both, human APP and PS1, in T cells from transgenic mice indicating that their lymphocytes can be used to study the effects of FAD mutations on cell death vulnerability or other cellular function. Human APP or PS1 revealed a similar expression pattern in mice as did endogenous APP or PS1 with a higher expression level in the brain than in non-neuronal cells (data not shown). A comparable expression pattern has also been found in man (Arai et al., 1991; Page et al., 1996).

Unchanged Pattern of Lymphocyte Subset Distribution in AD, But Age-Related Decrease of T Cells

Recently, we were able to show that peripheral lymphocytes exhibit an age-related increase in vulnerability to apoptosis compared to young controls (Schindowski et al., 2000). This enhanced susceptibility was even more pronounced in lymphocytes

from sporadic AD patients compared to nondemented aged controls (Eckert et al., 1998; Eckert et al., 2001a). Therefore, it was necessary to clarify whether these changes can be explained by the hypersensitivity of one specific lymphocyte subset only. To avoid misinterpretation of an enhanced apoptosis owing to changes in the distribution pattern of lymphocyte subsets with distinct sensitivity thresholds for the induction of cell death, we investigated the distribution of lymphocyte subsets in young subjects, nondemented aged controls, and AD patients.

The lymphocyte population mainly consists of T cells. Thus, changes in the percentage of T lymphocytes and/or their subsets may influence the total apoptotic balance in lymphocytes. In our present study, distribution of lymphocyte subsets was not altered among AD patients and nondemented controls (CD3⁺ cells [T cells]: $69.8 \pm 1.9\%$ in AD patients vs $66.0 \pm 2.2\%$ in controls; CD19⁺ cells [B cells]: 10.3 ± 0.85 in AD patients vs 10.9 ± 1.2 in controls). In contrast, the distribution pattern during aging is altered showing a slight decrease of T cells (CD3⁺ cells: $73.0 \pm 1.5\%$, $p < 0.01$ vs aged controls; CD19⁺ cells: $11.8 \pm 0.9\%$). To further investigate the distribution of T lymphocyte subpopulations, we counted the absolute cell number of CD3⁺ T cells as well as CD4⁺ and CD8⁺ T cells in the whole blood (Fig. 2A,B). Again, no differences were found between AD patients and nondemented aged controls, but a significant loss of CD3⁺, CD4⁺, and CD8⁺ T cells occurred during aging (Fig. 2C). However, CD4⁺ and CD8⁺ T-cell decline seems to be equally affected by aging, because CD4/CD8 ratio was unaltered. No differences in the distribution of lymphocyte and especially T-lymphocyte subsets were found in AD, thus we can exclude that this is a causative factor for the altered vulnerability to cell death found in the total population of peripheral lymphocytes from AD patients.

Evaluation of Apoptotic Cell Death in T-Lymphocyte Subsets

Next, we evaluated apoptotic cell death in T-lymphocyte subsets by flow cytometry analysis using instrument settings specific for the detection of apoptotic DNA fragments. The portion of apoptotic cells was quantified in CD4⁺ or CD8⁺ labeled

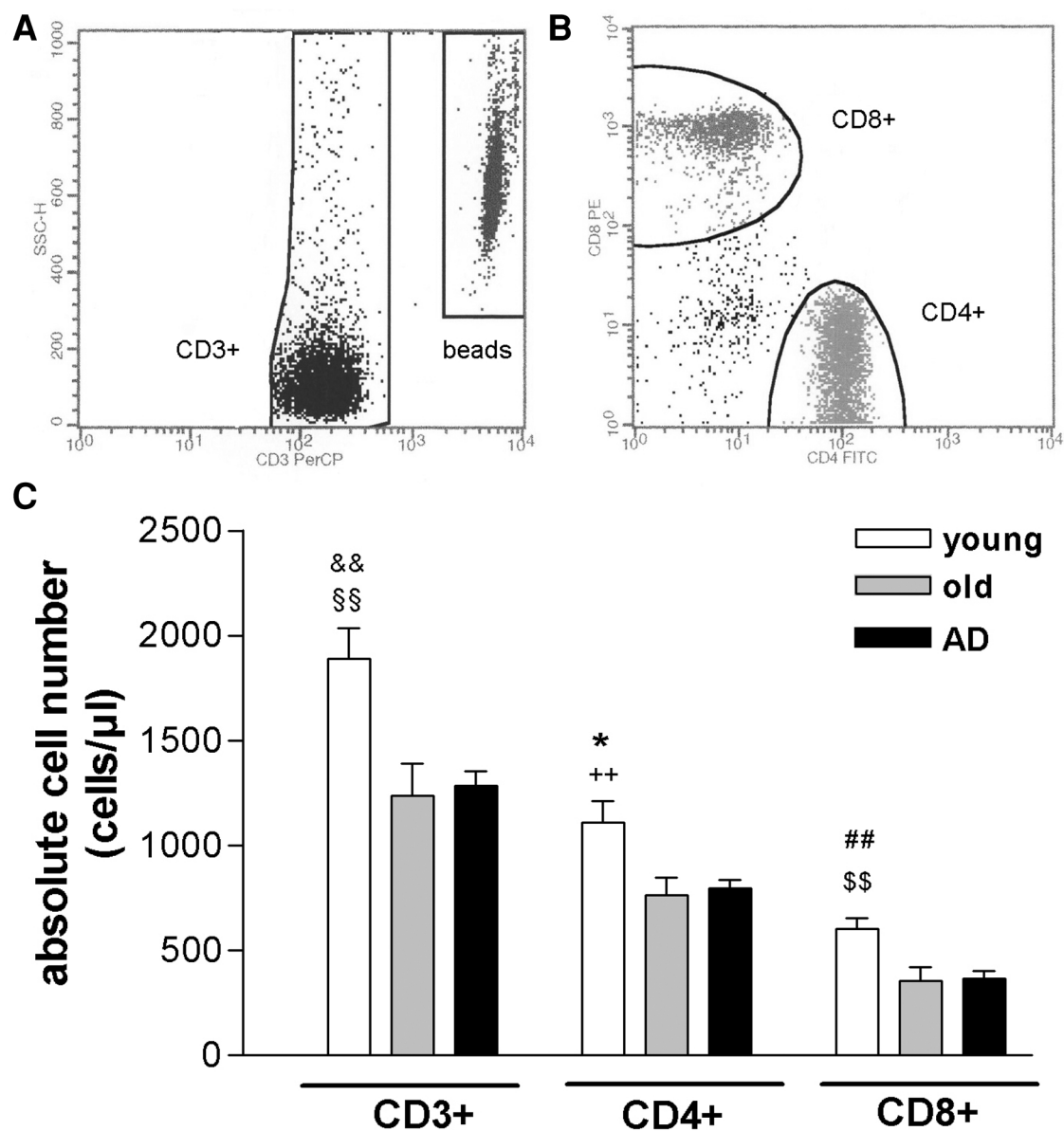
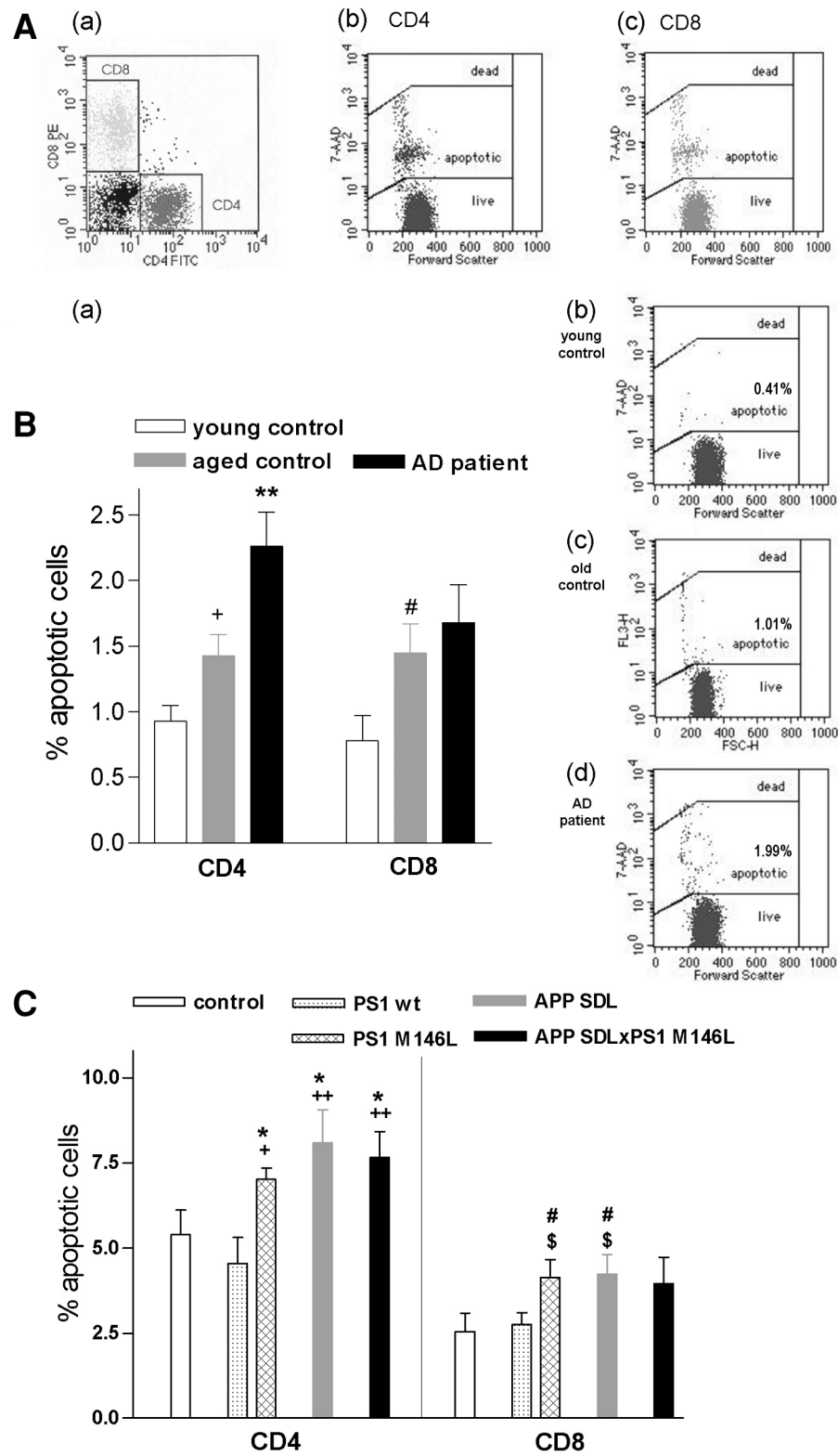


Fig. 2. Analyses of the composition of lymphocyte subsets. (A,B) Quantification of total lymphocyte number in whole blood using TrueCount[®]. Absolute number of lymphocytes was determined with TrueCount[®] beads (B,D) and triple-stained lymphocytes (CD3-PerCP, CD8-PE, and CD4-FITC). CD3⁺T cells were gated (A) and subsequent CD4⁺ and CD8⁺T cell analysis was only performed with CD3⁺ positive T cells (B) and related to bead counts. (C) Changes of T lymphocyte subsets in aging and AD. Absolute cell number of CD3⁺T cells plus CD4⁺ and CD8⁺T cells was determined in the whole blood. No differences were found between AD patients and nondemented aged controls, but a significant loss of CD3⁺, CD4⁺, and CD8⁺T cells in aging (ANOVA post hoc Tukey's test: CD3⁺: §§ $p < 0.01$ vs CD3⁺ from aged controls, && $p < 0.01$ vs CD3⁺ from AD patients; CD4⁺: ++ $p < 0.01$ vs CD4⁺ from aged controls, * $p < 0.05$ vs CD4⁺ from AD patients; CD8⁺: \$\$ $p < 0.01$ vs CD8⁺ from aged controls, ## $p < 0.01$ vs CD8⁺ from AD patients). Color image available for viewing at www.humanapress.com.



T cells using a co-staining technique with the fluorescent dye 7-AAD (Fig. 3A). We determined the accumulation of apoptotic cells under basal conditions or after 24 h in vitro incubation in the absence or presence of cell death triggering agents. For the activation of cell death, we used 2-deoxy-D-ribose (d-Rib), that is known to trigger apoptosis in lymphocytes by depletion of the intracellular pool of reduced glutathione thereby producing oxidative stress (Kletsas et al., 1998).

Basal In Vitro Apoptosis

To investigate the effects of aging and sporadic or genetic risk factors of AD on vulnerability to cell death in T-lymphocyte subsets, we studied isolated peripheral lymphocytes from young or aged nondemented controls and from sporadic AD patients on the one hand and lymphocytes from the spleens of transgenic mice bearing Alzheimer's mutations in PS1 and/or APP on the other hand. The latter model was used, because we already demonstrated that T cells from PS1 transgenic mice exhibited a similar hypersensitivity to cell death than did lymphocytes from AD patients (Eckert et al., 2001b). In addition, by investigating young mice,

we can avoid potential overlapping effects as a result of aging.

In flow cytometry analysis, freshly isolated T cells from aged controls showed a significant increase in basal in vitro apoptosis of both, CD4⁺ as well as CD8⁺ T cells, compared to the T-cell subsets of young control subjects (Fig. 3B). In contrast, AD patients showed a highly significantly enhanced basal in vitro apoptosis only of CD4⁺ T cells when compared to cells from nondemented aged control subjects (Fig. 3B). CD8⁺ T cells from AD patients showed a similar, but not significant tendency with regard to slightly elevated levels of apoptotic cells when compared to control cells (Fig. 3B). In parallel, CD4⁺ T cells from mice expressing mutant PS1 (PS1 M146L) and/or mutant APP (APP695 SDL) exhibited significantly enhanced levels of apoptotic cells compared to CD4⁺ T cells from PS1 wt Tg and nontransgenic controls (Fig. 3C). The same effects could be additionally observed in CD8⁺ T cells from transgenic mice bearing mutant APP or PS1, even though the maximum of the apoptotic proportion in CD8⁺ T cells was less than in CD4⁺ T cells from all groups of mice. There was no significant difference between CD8⁺ T cells from double transgenic APP/PS1 mice and control mice. This is probably because of the

Fig. 3. (see opposite page) Detection of apoptosis in lymphocyte subsets by flow cytometry and Alzheimer-related changes of basal in vitro apoptosis (A) Determination of apoptotic cells with triple staining using 7-AAD and monoclonal antibodies against T lymphocyte subsets CD4 and CD8. CD4 (FL-1 channel) and CD8 (FL-2 channel) positive cells (a), respectively, were gated and displayed in a new dot plot showing 7-AAD fluorescence vs forward scatter light (b,c). Living, early and late apoptotic cells were defined according to the level of 7-AAD staining (labeled "live, apoptotic and dead" within dot plots). (b) shows the determination of apoptotic cells of gated CD4⁺ T lymphocytes, (c) shows the determination of apoptotic cells of gated CD8⁺ T lymphocytes. (B) (a) Basal apoptosis in CD4⁺ T cells and in CD8⁺ T cells from young subjects ($n = 11$), elderly nondemented controls ($n = 21$), and AD patients ($n = 26$). CD4⁺ T lymphocytes from AD patients showed significantly elevated levels of basal apoptotic cells compared to nondemented controls (ANOVA post hoc Tukey's test: $**p < 0.01$ vs CD4⁺ from young and aged controls). Basal apoptosis was elevated in CD4⁺ T cells as well as in CD8⁺ T cells from aged controls compared to young ones (ANOVA post hoc Tukey's test: $+p < 0.05$ vs CD4⁺ from young controls, $\#p < 0.05$ vs CD8⁺ from young controls). Two-way ANOVA revealed significant differences between groups ($p < 0.001$). (b-d) Representative dot blots of individual subjects showing determination of basal apoptosis by 7-AAD measurement in CD4⁺ T cells from young control (b), aged control (c), and AD patient (d). (C) Basal apoptosis in CD4⁺ T cells and in CD8⁺ T cells from nontransgenic controls and from transgenic mice expressing either mutant human APP (APP695SDL) or mutant human PS1 (PS1 M146L), or both, or human wild-type PS1 (PS1 wt) ($n = 6$ for all groups). T cells bearing FAD-associated mutations displayed significantly elevated levels of basal apoptotic cells compared to PS1 wt or nontransgenic controls (ANOVA post hoc Tukey's test: $*p < 0.05$ vs CD4⁺ from control; $+p < 0.05$, $++p < 0.01$ vs CD4⁺ from PS1 wt, $\#p < 0.05$ vs CD8⁺ from control; $\$p < 0.05$ vs CD8⁺ from PS1 wt, $n = 6$). The absolute increase in apoptosis was higher in CD4⁺ T cells than in CD8⁺ T cells: two-way ANOVA revealed significant differences between mice groups ($p < 0.001$) and the lymphocyte subsets ($p < 0.001$).

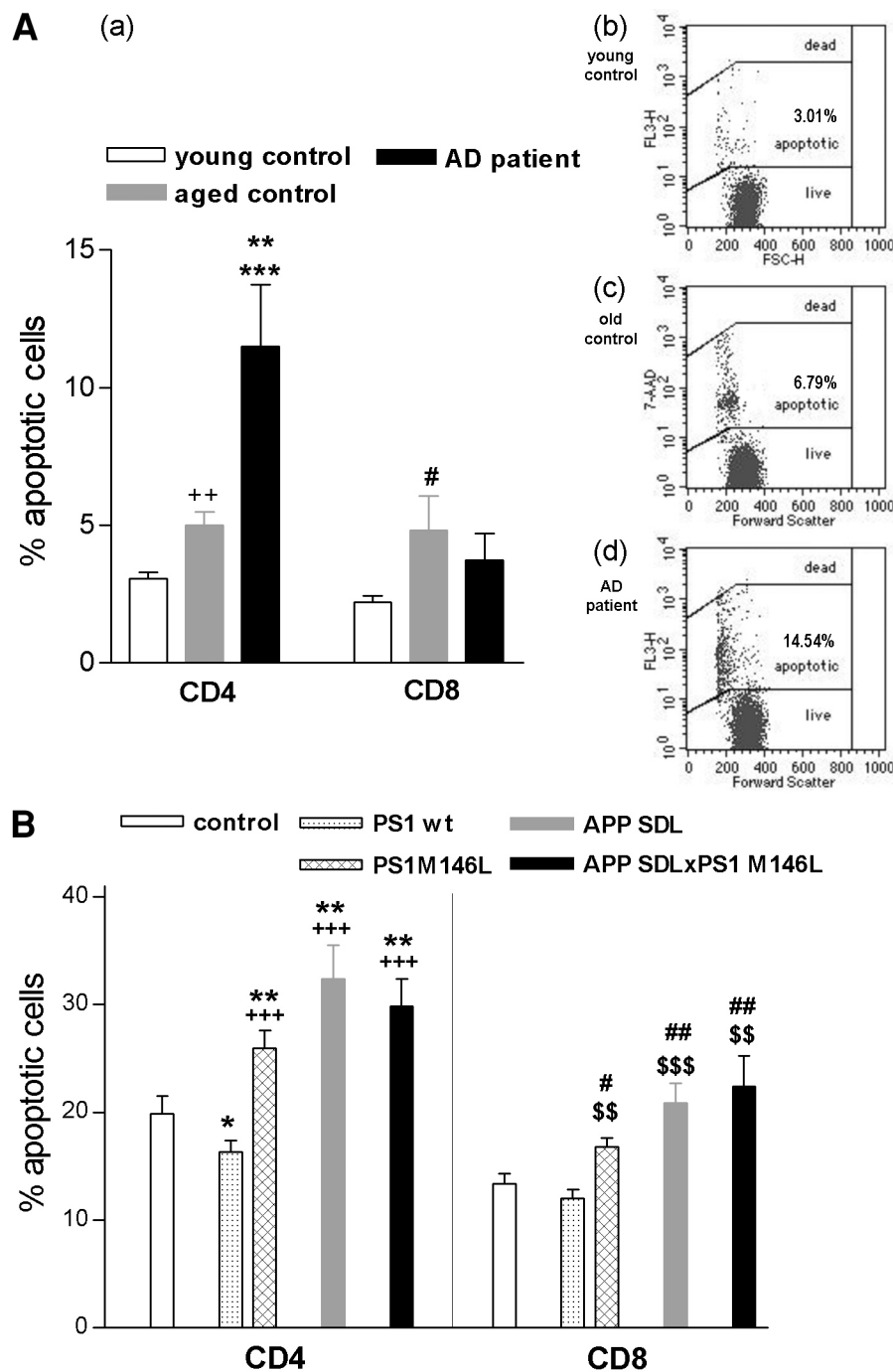


Fig. 4. Alterations of spontaneous apoptosis in aging and Alzheimer's disease (A) Spontaneous in vitro apoptosis in CD4⁺ T cells and in CD8⁺ T cells from young subjects ($n = 11$), nondemented controls ($n = 12$), and AD patients ($n = 12$) after 24 h in vitro incubation. Again, CD4⁺ T cells from AD patients showed a highly significantly increased spontaneous apoptosis compared to control subjects (ANOVA post hoc Tukey's test: $**p < 0.01$ vs CD4⁺ from aged controls; $***p < 0.001$ vs CD4⁺ from young controls), whereas CD8⁺ T cells did not differ. Spontaneous apoptosis was elevated in CD4⁺ T cells as well as in CD8⁺ T cells from aged controls compared to young ones

high variance of apoptotic levels in APP/PS1 T lymphocytes within this experiment.

Taken together, these findings indicate that especially the increased susceptibility of CD4⁺ T cells from sporadic AD patients as well as from FAD transgenic mice mainly contributes to the total vulnerability of T lymphocytes, whereas the hypersensitivity of both cell types appears to participate in the total vulnerability of aged lymphocytes.

Spontaneous In Vitro Apoptosis

Next, we studied apoptosis after 24 h in vitro incubation termed spontaneous apoptosis. Again, apoptosis was increased in CD4⁺ T cells as well as in CD8⁺ T cells from aged controls compared to young ones (Fig. 4A). Notably, also in this paradigm, exclusively CD4⁺ T cells from AD patients showed a highly significantly enhanced spontaneous apoptosis compared to CD4⁺ T cells from nondemented aged control subjects (Fig. 4A), whereas CD8⁺ T cells from AD patients did not (Fig. 4A). Similarly, CD4⁺ T cells from mice expressing mutant PS1 (PS1 M146L) and/or mutant APP (APP695SDL) exhibited significantly enhanced basal levels of apoptotic cells compared to CD4⁺ T cells from PS1 wt Tg and nontransgenic littermate controls (Fig. 4B). Also under these conditions, increased apoptosis was identified in CD8⁺ T cells from transgenic mice bearing mutant APP and/or PS1 (Fig. 4B). Again, CD8⁺ T cells exhibited apoptosis to a lesser degree as did CD4⁺ T cells from all groups of mice confirming our observations of the apoptotic behavior under basal conditions (Fig. 3C).

Oxidative Stress-Induced Apoptosis

To investigate the induction of apoptosis under conditions mimicking oxidative stress, we stimulated T lymphocytes with d-Rib for 24 hr in vitro. Again, apoptosis was increased in CD4⁺ T cells as well as in CD8⁺ T cells from aged controls compared to young ones (Fig. 5A). There was a slight, nonetheless cell type-specific increase in d-Rib-induced apoptosis in CD4⁺ T cells from sporadic AD patients compared to nonaffected controls that was not present in CD8⁺ T cells. However, this effect failed to reach statistical significance ($p = 0.06$) (see Fig. 5A). Expression of mutant APP and/or PS1 in CD4⁺ T cells resulted in a significantly increased levels of apoptotic cells after exposure to oxidative stress compared to CD4⁺ T cells from PS1 wt Tg and nontransgenic controls (Fig. 5B), whereas CD8⁺ T cells reacted again much less sensitive (Fig. 5B). These findings further emphasize the cell type-specific sensitivity of CD4⁺ T cells to the induction of apoptotic cell death in AD.

Discussion

Enhanced vulnerability to cell death appears to be an important factor of aging and age-related sporadic AD. Although, histological studies in post-mortem AD brain tissue indicate enhanced features of apoptotic cell death, such as DNA fragmentation (Cotman, 1998; Stadelmann et al., 1999), evidences for an enhanced cellular vulnerability to the induction of apoptosis primarily originate from studies

Fig. 4. (continued) (ANOVA post hoc Tukey's test: $++p < 0.01$ vs CD4⁺ from young controls, $\#p < 0.05$ CD8⁺ from young controls). Two-way ANOVA revealed significant differences between groups ($p < 0.001$) and the lymphocyte subsets ($p < 0.01$; AD CD4⁺ vs AD CD8⁺: $p < 0.01$). (b–c) Representative dot blots of individual subjects showing determination of spontaneous in vitro apoptosis by 7-AAD measurement in CD4⁺ T cells from young control (b), aged control (b), and AD patient (d). (B) Spontaneous in vitro apoptosis in CD4⁺ T cells and in CD8⁺ T cells from nontransgenic controls and from transgenic mice expressing either mutant human APP (APP695SDL) or mutant human PS1 (PS1 M146L), or both, or human wild-type PS1 (PS1 wt) ($n = 6$ for all groups). Both, CD4⁺ and CD8⁺ T cells, bearing FAD-associated mutations displayed significantly elevated levels of apoptotic cells after 24 h incubation compared to PS1 wt or nontransgenic controls (ANOVA post hoc Tukey's: $*p < 0.05$, $**p < 0.01$ vs CD4⁺ from control; $++p < 0.01$, $+++p < 0.001$ vs CD4⁺ from PS1 wt; $\#p < 0.05$, $##p < 0.01$ vs CD8⁺ from control; $$$$p < 0.01$, $$$$$p < 0.001$ vs CD8⁺ from PS1 wt, $n = 6$). Again, apoptosis was more pronounced in CD4⁺ cells: two-way ANOVA revealed significant differences between mice groups ($p < 0.001$) and the lymphocyte subsets ($p < 0.001$).

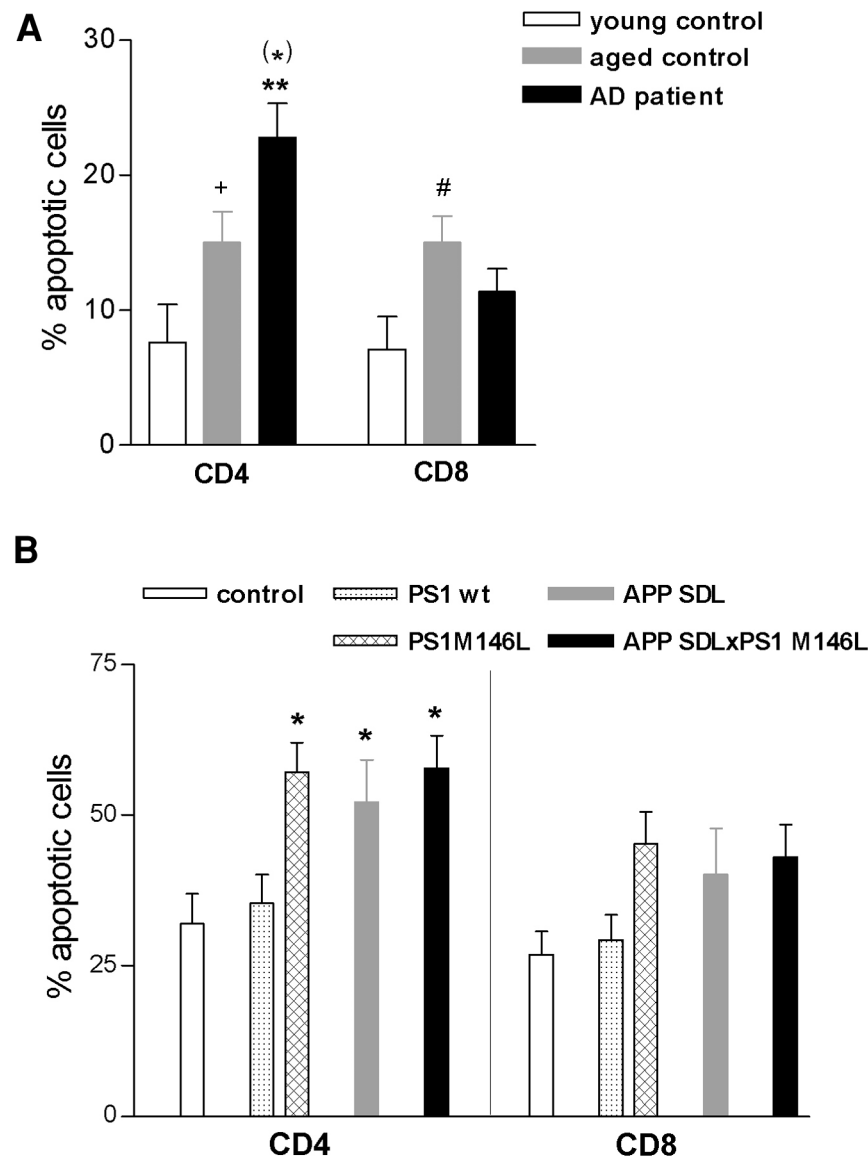


Fig. 5. Changes of oxidative stress-induced apoptosis in aging and Alzheimer's disease. **(A)** Apoptosis in CD4⁺ T cells and in CD8⁺ T cells from young subjects ($n = 11$), nondemented controls ($n = 12$), and AD patients ($n = 12$) after exposure to oxidative stress. Cell death was triggered in vitro by treatment with d-Rib (10 mM) for 24 h. Apoptotic cell death in CD4⁺ T cells from AD patients tended to higher levels compared to nondemented aged controls (ANOVA post hoc Tukey's test: $**p < 0.01$ vs CD4⁺ from young controls; $(*)p = 0.06$ vs CD4⁺ from aged controls, student's t -test), whereas apoptosis in CD8⁺ T cells did not differ. d-Rib-induced apoptosis was elevated in CD4⁺ T cells as well as in CD8⁺ T cells from aged controls compared to young ones (ANOVA post hoc Tukey's test: $+P < 0.05$ vs CD4⁺ from young controls, $\#p < 0.05$ vs CD8⁺ from young controls). Two-way ANOVA revealed significant differences between groups ($p < 0.01$) and the lymphocyte subsets ($p < 0.05$; AD CD4⁺ vs AD CD8⁺: $p < 0.01$). **(B)** Apoptosis in CD4⁺ T cells and in CD8⁺ T cells from nontransgenic controls and from transgenic mice expressing either mutant human APP (APP SDL) or mutant human PS1 (PS1 M146L), or both, or human wild-type PS1 (PS1 wt) after exposure to oxidative stress ($n = 6$ for all groups). Cell death was triggered in vitro by treatment with d-Rib (10 mM) for 24 h. CD4⁺ T cells bearing mutant PS1, and/or APP displayed significantly elevated levels apoptotic cells compared to PS1 wt or nontransgenic controls (ANOVA post hoc Tukey's test: $*p < 0.05$ vs CD4⁺ from control, $n = 6$). In CD8⁺ T cells from the same transgenic mice, a similar tendency to elevated apoptosis could be observed, again to a lesser extent when compared to the corresponding CD4⁺ T cells: two-way ANOVA revealed significant differences between mice groups ($p < 0.001$) and the lymphocyte subsets ($p < 0.01$).

using lymphocytes from sporadic AD patients (Parshad et al., 1996; Eckert et al., 1998; Mecocci et al., 2002). The use of these easily accessible cells from AD patients complements studies of autopsy samples in a meaningful matter and provides a useful tool to investigate dynamic processes such as the regulation of cell death pathways, signal transduction mechanisms, intracellular calcium regulation, and oxidative metabolism (Mecocci et al., 1994; Gibson et al., 1996; Eckert et al., 1998; Gibson et al., 2000; Mattson et al., 2001; Eckert et al., 2001a; Gibson et al., 2002; Mecocci et al., 2002). Abnormalities in each of these processes occur in AD and can be linked to neuronal death and brain dysfunction. Some of these pathological changes are also related to aging and are additionally altered in AD by common or divergent mechanisms (Quadri et al., 1996; Poulin et al., 1996; Eckert et al., 1997a; Eckert et al., 1997b; Schindowski et al., 2000). Cell culture experiments also suggest that defects in the cellular calcium stores, ability to handle oxidative stress, and to respond to metabolic impairment, link the FAD-causing PS1 mutations to the disease process (Mattson et al., 2001). Importantly, our recent findings in lymphocytes from PS1 mutant transgenic mice mirror comparable findings in lymphocytes from AD patients (Parshad et al., 1996; Eckert et al., 1998; Eckert et al., 2001a) indicating that disturbances in the metabolism of ROS inside cells, abnormal calcium homeostasis, and increased susceptibility to cell death may contribute to the pathogenic mechanisms of PS-1 mutations in vivo. Accordingly, evidence supports a similarly increased vulnerability to oxidative stress-induced DNA damage present in lymphocytes from FAD patients bearing PS1 mutations (Parshad et al., 1996).

Because a difference in the distribution pattern of lymphocyte and particularly T lymphocyte subsets between AD patients and nondemented controls was not observed, it can be concluded that CD4⁺ T cells mostly contribute to the increased apoptotic cell levels found in peripheral lymphocytes from AD patients (Eckert et al., 1998; Eckert et al., 2001a). This defect itself appears not to be sufficient to lead to a pathological leukopenia in AD patients probably owing to the rapid restoration process of deleted lymphocytes, a balanced regulation that is clearly not possible for the affected brain cells. Whereas in AD brain the accumulation of A β and oxidative damage put neuronal cells under chronic

stress for many years, the lack of these accumulation processes in the periphery may also explain why massive cell loss is not observed in circulating blood cells or fibroblasts.

One major concern regarding the hypothesis of common alterations in the brain and immune system relates to the observation that the spatial patterns of lesions in AD brain may reflect pathological processes affecting particular neuroanatomical structures or susceptible neuronal subpopulations, whereas other neuronal subgroups are strikingly resistant to cell death (Armstrong et al., 2001).

In the present study, we clearly demonstrate that in the immune system specific subpopulations of the same cell type are more vulnerable to cell death than others. CD4⁺ T cells from sporadic AD patients exhibited strong disease-specific alterations in the vulnerability to cell death compared to CD4⁺ T cells from aged nondemented controls, whereas no differences in the susceptibility of CD8⁺ T cells to apoptosis have been found for all conditions investigated. As demonstrated previously, our assay investigating vulnerability of lymphocytes to cell death appears to possess specificity for AD, because patients with vascular dementia did not show an increased susceptibility to apoptosis (Eckert et al., 2001a). However, there is some overlap in apoptotic levels of CD4⁺ cells between AD patients and controls. Therefore, it would be very interesting to investigate the risk for AD of these subjects during a longitudinal study.

Consistent with this observation, FAD mutations also increase vulnerability of CD4⁺ T cells to apoptosis. The same tendency was evident in CD8⁺ T cells but to a lesser degree. Thus, sporadic risk factors of AD exhibit the highest impact on cell type-specific increase in the sensitivity of CD4⁺ T cells to cell death. Nevertheless, genetic factors preferentially impact the same specific cell type as well as do sporadic factors. In contrast, the age-related elevation of apoptotic cell death was not cell type-specific, given that increased apoptosis was characteristic for both, CD4⁺ and CD8⁺ T cells from aged controls. Only age-related enhanced vulnerability of CD4⁺ T cells can be further heightened by AD but not that of CD8⁺ T cells (Schindowski et al., 2002). Aging represents the most important risk factor for sporadic AD, thus, it may also directly contribute to the same final pathway in CD4⁺ T cells.

Why do CD4⁺ T cells from AD patients exhibit this cell type-specific increase in the vulnerability

to cell death? Several experimental studies provide support for an AD-specific cause-effect relationship between increased susceptibility of CD4⁺ T cells and other pathological changes probably involved in cellular dysfunction and cell death. For example, our results support findings that serum levels of CD95 (Fas, Apo-1), known to mediate apoptosis, are elevated in AD patients (Richartz et al., 2002). Indeed, preliminary findings of our group indicate that CD4⁺ T cells from AD patients are more vulnerable to the induction of apoptosis by Fas-ligand (FasL) than CD4⁺ T cells from aged nondemented controls (AD: 25.8% \pm 7.0% vs Co 12.2 \pm 2.1%, n = 6; p < 0.05). In particular, there seems to be a significant increase in Fas expression in CD4⁺ T cells of AD patients (Lombardi et al., 1999) that may explain their cell type-specific increased vulnerability to apoptosis. Interestingly, these findings are also in agreement with data demonstrating that Fas is involved in inflammatory processes in AD brain. Thus, increased soluble Fas concentrations have been found in the cerebrospinal fluid from AD patients compared with nondemented controls (Martinez et al., 2000). The accumulation of A β may also lead to cross-linking and activation of death receptors, such as Fas, resulting in caspase activation and neuronal cell death in AD brain (Ivins et al., 1999). In addition, Fas antigen is expressed by a subset of reactive astrocytes in AD patients and may play a pivotal role in governing astrocyte apoptotic or inflammatory responses (Saas et al., 1999). Moreover, growing evidence suggests that mutant PS1 and/or mutant APP sensitize cells to apoptosis, by linking perturbed calcium homeostasis to the pathogenic action of AD-specific mutations. Again, these changes are not limited to neurons (Mattson et al., 1993; Parshad et al., 1996; Sulger et al., 1999; Mattson et al., 2001; Eckert et al., 2001b; Eckert et al., 2001c). Interestingly, different mutations in both genes, APP and PS1, appear to have the same final effect of an increased vulnerability to cell death in lymphocytes. Our experiments did not support a more pronounced vulnerability in CD4⁺ T cells from double transgenic mice compared to mice bearing either PS1 or APP alone. These results provide support to our hypothesis that PS1 and APP mutations affect the same common final pathway in lymphocytes, rather than different ones, because in the latter case an additive effect of both genes would be expected. Given that lymphocytes from transgenic

mice showed alterations in the vulnerability to the induction of apoptosis when the animals did not exhibit an obvious neurodegeneration or A β plaques, the alterations described above may occur independently from or may precede AD brain pathology. Additionally, our results of an increased cell type-specific vulnerability are in accordance with an altered calcium regulation in CD4⁺ T lymphocytes from familial (PS1 mutations) and sporadic AD patients compared to lymphocytes from age-matched controls (Grossmann et al., 1993), confirming the implication of this defect for all cases of AD. Therefore, the altered calcium response in CD4⁺ T cells from AD patients may be related to increased vulnerability of CD4⁺ T lymphocytes from AD patients to apoptosis. Calcium mediates responses of lymphocytes to a variety of signals, thereby regulating cell proliferation, differentiation, and cell death. It remains to be determined how increased Fas receptor expression, circulating factors like Fas and A β (Eckert et al., 1993), and disturbed calcium regulation in CD4⁺ T cells from AD patients might result in an increased vulnerability to apoptosis of these cells. It seems reasonable that AD-related pathogenic mechanisms are directly linked to the cell-type specific change in the sensitivity threshold.

Changes in immune cell function in AD patients, and animal models suggest the existence of an immunological contribution to the pathogenesis and progression of AD. The immune system normally plays an important role in removing toxic A β from the brain as recently shown by the immunization of transgenic mice with aggregated forms of A β (Schenk et al., 1999). Human autoreactive lymphocytes can recognize A β and might therefore be important for its elimination (Marx et al., 1998). In addition to cytotoxic CD8⁺ T lymphocytes, a population of CD4⁺ T lymphocytes exists that has an immunomodulatory role, expresses cytolytic activity (CD4⁺ CTL) after activation, and induces apoptosis in Fas-positive target cells (Hahn et al., 1995). Activated T lymphocytes can readily cross the endothelial blood-brain barrier. Having crossed the blood-brain barrier, the activated T cells interact with local glial cells by releasing factors, including interferon-gamma and tumor necrosis factor alpha, before undergoing apoptosis. Notably, recent findings demonstrate an adverse effect of PS1 mutations on microglial cells resulting in their hyperactivation under pro-inflammatory conditions, which

may, together with direct effects of mutant PS1 in neurons, contribute to the neurodegenerative process in AD (Lee et al., 2002). Thus, the immune surveillance of the central nervous system seems to be implemented by activated T cells. The findings of the present study may be of potential relevance to the understanding and design of an effective immunological approach to the diagnosis and prevention of AD.

Taken together, our data support common but also divergent mechanisms of an enhanced susceptibility of lymphocytes to apoptosis in aging and AD. It remains to be determined exactly which defect finally results in the cell-type specific sensitivity of AD lymphocytes. Our findings further emphasize the central role of enhanced cellular vulnerability to cell death as a common pathological pathway for sporadic and familial AD with aging possibly playing a contributory role (Mattson, 2000).

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